Rec'd PCT/PTO 28 FEB 2000

FORM PTO-1390 (REV 11-98) — U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: SCBREV-223

U.S. APPLICATION NO (If known, see 37 CFR 1 5): 09/486660

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IT98/00231	11 August 1998	28 August 1997

TITLE OF INVENTION: TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS

APPLICANT(S) FOR DO/EO/US: SACCO, Maria Grazia; ZECCA, Luigi; BROMLEY, Peter; RONCUCCI, Romeo (Deceased); CLERICI, Libero A.; and VEZZONI, Paolo

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2)) (Publ. No. WO 99/11772)
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article (35 U.S.C. 371(c)(5)).

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Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

(A) Formal Drawings - Four (4) Sheets containing Figs. 1A-C and 2-4;
(B) Copy of PCT Request, as filed;
(C) Copy of International Search Report, including references cited therein;
(D) Copy of International Preliminary Examination Report;
(E) Copy of Letter dated 11 February 2000 to the International Bureau of WIPO from applicants' Italian attorneys identifying the heirs of Romeo RONCUCCI, Deceased;
(F) The addresses of the applicants are as stated on the attached unexecuted Declarations.

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U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/486660	INTERNATIONAL APPLICATION NO PCT/IT98/00231	ATTORNEY'S DOCKET NUMBER SCBREV-223

	CALCULATIONS PTO USE ONLY	
17. [X] The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$760.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ -0-	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	12 - 20 =	0	X **$18.00**	$ -0-**	
Independent claims	1 - 3 =	0	X **$78.00**	$ -0-**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.00**	$ -0-**	
TOTAL OF ABOVE CALCULATIONS =				$ 840.00**	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$ -0-	
SUBTOTAL =				$ 840.00**	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ -0-	
TOTAL NATIONAL FEE =				$ 840.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ -0-	
TOTAL FEES ENCLOSED =				$ 840.00**	
Amount to be: refunded				$	
charged				$	

****Filing Fee calculated after entry of the Preliminary Amendment filed herewith which deletes multidependencies.**

a. [X] A check in the amount of $ 840.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 07-2153. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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28 February 2000

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: SACCO, Maria Grazia, et al Serial No. _____ (based on PCT/IT98/00231 filed on 11 August 1998) Filed: Herewith For: TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS	Atty. Docket: SCBREV-223 Date: 28 February 2000

PRELIMINARY AMENDMENT

BOX: PCT (DO/EO/US)
Assistant Commissioner for Patents
Washington, D. C. 20231

Sir:

Prior to calculating the filing fee, please amend the above-identified application as follows:

IN THE CLAIMS:

In the claims as amended during prosecution of the corresponding international application, i.e. PCT/IT98/00231 (copy attached), please amend the claims as follows:

Claim 5, line 3, change "claims 1-4" to --claim 1--.

Claims 7, 8 and 9, line 1 of each, change "claims 5-6" to --claim 5--.

R E M A R K S

With the above amendments, the multidependencies of claims 5 and 7-9 have been deleted.

Respectfully submitted,
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CLAIMS

1. A non-human transgenic mammal which comprises cells containing a construct of a heat shock protein (hsp) promoter linked to the growth hormone (GH) gene sequence.

2. A non-human transgenic mammal according to claim 1, wherein the heat shock protein promoter is hsp70 gene promoter.

3. A non-human transgenic mammal according to claim 1, which is a rodent.

4. A non-human transgenic mammal according to claim 3, which is a mouse.

5. A method for the study of chemical, physical and biological toxic agents which comprises:

a) exposing the transgenic mammal of claims 1-4 to the toxic agent;

b) determining the effect through measurement of the hematic concentration of the reporter-gene.

6. A method according to claim 5, wherein the same animal is used for repeated tests with the same or different toxic agent.

7. A method according to claims 5-6, for the study of toxicity kinetics of one or more toxic agents.

8. A method according to claims 5-6, for the study of heat stress.

9. A method according to claims 5-6, for the study of metal toxicity.

10. A method according to claim 9 for the study of toxicity of metals selected from the group consisting of Rb, Cu, Hg, As and Cd.

11. The use of the transgenic mammal of claim 1 for in vivo toxicity studies.

12. The use of a transgenic animal according to claim 11, wherein said animal is a mouse.

4/PRTS

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PCT/IT98/00231

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TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS

The present invention provides transgenic animals for the study of biological, physical and chemical toxic agents.

At present, toxicity tests can be carried out both in vivo and in vitro.

The industrials, the public opinion and the scientific community are strongly interested in the abolition of toxicity tests made on animals and therefore in their replacement with in vitro tests.

This target, however, is quite unrealistic at the moment, since no in vitro tests which can replace in vivo tests are available, either now or in the near future.

It is well known, in fact, that the substances under in vivo investigation often undergo metabolic modifications, which might significantly alter their toxicity profile, to an extent which would be unpredictable in in vitro tests.

On the other hand, in vivo studies always involve animal suffering and sacrifice.

However, it is possible to conceive genetically-engineered animal models which may simplify the determination of the toxicity of various agents and reduce the number of animals involved.

Recently, the use of transgenic animals as models for pharmacological studies has been proposed.

For example, EP 0 169 672 B1 describes transgenic animals bearing oncogenes like c-myc, suitable for the

study of tumors associated to the expression of such oncogenes, or bearing the human growth hormone gene fused to a metallothionein promoter, whereby, said promoter being an inducible promoter, it is possible to study the effect of the expression, upon induction, of the associated gene on the whole organism (Palmiter et al. (1983) Science 222, 809).

WO 91/15579 describes a method for studying mutagenesis in transgenic animals bearing DNA sequences which can easily be extracted and analysed for mutations.

The present invention provides non-human transgenic animals useful for toxicity studies.

Such animals are characterised in that they have regulatory DNA sequences in some or all their cells, which are sensitive to biological, physical and chemical toxic agents, functionally linked to sequences of reporter genes, whereby the expression of the latter sequences is controlled or induced by said regulatory sequences.

Among the regulatory sequences, the stress-promoter sequences, like the heat shock protein (hsp) promoters, are preferred, but also cytochrome-promoters of the p450-superfamily, as well as those promoters of other genes, like p53 gene, activated by biological, chemical or physical stress, can be cited.

Among suitable reporter genes, the growth hormone gene, which has been used in the experiments described below, is preferred, but also chloramphenicol acetyl transferase (CAT), green fluorescence protein (GFP) and β-galactosidase (LacZ) genes can be suitably employed.

The transgenic animals of the invention can be used

in a method for studying the toxicity induced by various agents.

In theory, any animal normally suitable for a toxicity test can be used in the method of the invention. In practice, non-human mammals, particularly primates and rodents, are preferred.

Mice, in particular, are the most preferred.

Conventional methods can be used for the production of transgenic animals, including, for example, the microinjection of recombinant DNA into embryonal cells or into pronuclei of one-cell stage embryos, the zygote, embryo cell, somatic cell or animal tissue infection with a virus, in particular with a retrovirus, according to what described, for example, in Hogan et al., Cold Spring Harbor Laboratory Press, NY, 1986; Palmiter et al., Ann. Rev. Genet., 20: 465-499; 1986; Capecchi, Science, 244: 288-292, 1989.

The method for the in vivo assay of potential toxic compounds according to the present invention, comprises exposing the animal to a chemical or physical agent for a time sufficient to induce the effect, and simply measuring the reporter gene expression. When the reporter gene encodes a protein secreted in the bloodstream, for instance, its hematic concentration, as well as other chemical-clinical parameters associated with the effect caused by the activation of the stress promoter, could be detected.

According to the first aspect of the invention, a preferred embodiment is the production of transgenic mice in which a construct has been inserted, which comprises a hsp promoter fused to growth hormone (GH)

gene (transgene), said promoter being described in Dreano et al. (Biotechnology 6:953, 1988 and Gene 49:1-8, 1986) and in Fishbach et al. (Cell Biol. Toxicol. 9:177-188, 1993). The latter publication reports that the exposure to toxic metals of a stable fibroblast line, engineered with a construct containing the growth hormone gene under the control of hsp promoter, causes the secretion of the reporter gene in the medium.

According to the preferred embodiment of the invention, the injury caused by the toxic agent is determined as the increase of GH plasma concentration versus the control.

This model has resulted particularly efficient and sensitive, especially in relation with toxic metals, but it can suitably be used also for other classes of chemical toxic compounds, like endocrine disruptors, as well as for other physical or chemical agents, like radiations and electromagnetic fields.

The main advantages offered by the invention are: the possibility to diminish animal suffering, since only low amounts of the test substances are used, surely lower than the dosages which could induce animal suffering or death; the reduction of the number of animals used in toxicological tests; the provision of a model that is absolutely reliable for what concerns the metabolic modifications, which the toxic agents undergo in the organism, the interactions of toxic compounds with various organs and their final effects on cells, including the chronic effects. This model is particularly useful for test reiterations and allows to monitor the agent's effect during long-lasting

treatments using always the same animal, thus eliminating the variability of the individual response. Further, several compounds can be studied using the same animal. Finally, such transgenic models can be used also for in vivo studies of toxicity kinetics of toxic compounds.

The second aspect of the invention concerns the possibility to obtain primary cultures of cells from different tissues of the transgenic animal, in which a recombinant DNA construct is integrated as described above, whereby a cell- or tissue-specific toxicity study can be carried out and the intracellular biochemical effects connected to toxicity can be evaluated under controlled conditions and in more detail during different stages of animal growth.

In this case, the in vitro assay comprises preparing primary cultures in conditions variable depending on the cell type, exposing said cultures to the toxic agent and monitoring the activation of the stress promoter through detection of the protein encoded by the reporter gene.

Referring to the above described transgenic mice bearing the hsp/GH construct, an embodiment of the second aspect of the invention consists for example in preparing primary cultures of fibroblasts, kidney, lung or bone marrow cells, hepatocytes or other, in their simultaneous or separate treatment with one or more toxic agents, and in the determination of GH secretion in the medium.

If, using the above assay, a tissue or a cell-type resulted sensitive to the toxic agent, a deeper

biochemical analysis could be made in order to find which cellular pathways are particularly involved in the toxicity.

Thus, according to a further aspect, the invention provides a method to carry out in vitro toxicity tests on primary cultures of somatic cells derived from a transgenic animal.

BRIEF DECRIPTION OF THE FIGURES

Fig 1. Panel A: Southern blot analysis of transgenic heterozygous (lanes 1-4) and homozygous mice (lanes 5-7) and a non-transgenic control mouse (lane 8).

Panel B: RT-PCR with hGH specific primers of heat-shock activated liver cells from transgenic mice. Samples: RNA from cultured hepatocytes before (lane 1) and 30 min after (lane 2) heat shock in vitro; RNA from livers before (lane 3) and 30, 60, 90, minutes after heat shock (lanes 4-6). + and - represent the negative and positive controls respectively. Lanes 7 to 10 are the amplifications on non-retrotranscribed liver RNAs performed on the same samples as in lanes 3 to 6. M1: marker V, M2: 1 kb ladder.

Panel C: RT-PCR with HPRT specific primers performed on RNAs from the samples 1 to 6 as in panel B.

Fig. 2: Plasma levels of hGH (pg/ml) measured at different times in transgenic mice after thermal stress. Values represent the mean ± SE; the number of mice tested for each time period is indicated by the number above each bar.

Fig. 3: Mean hGH plasma levels (pg/ml) ± SE observed in transgenic mice injected i.p. with PBS and with various inorganic toxic compounds at the indicated

doses. Besides controls, are indicated: Rb: rubidium chloride; Hg: methylmercurium chloride; Cu: copper sulphate; Cd: cadmium chloride; As: sodium arsenite (2 doses)(below each bar is given the number of tested mice). The levels of significance are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.005$

Fig. 4: Mean ± SE of plasma hGH levels observed in transgenic mice subjected to two consecutive treatments, according to the following schema:

Group	First treatment (T_1)	Second treatment (T_2)	Time Interval (T_1-T_2)
As_1	As	As	10 days
As_2	Cd	As	2 months
As_3	Rb	As	2 months
Cu	Cu	Cu	2 months
Control	untretated	untreated	

The following examples better illustrate the invention:

EXAMPLE 1

Production and characterization of a transgenic mouse lineage

Transgenic mice were produced according to standard techniques (Hogan et al., "Manipulating the mouse embryo: a laboratory manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986), by microinjecting 1-cell stage embryo pronuclei with a 1.4 kb EcoRI DNA fragment from p17hGH construct (described in Dreano et al., Biotechnology 6:953, 1988 and Gene

49:1-8, 1986), containing the human growth hormone cDNA as reporter gene, fused to the control region of the human Hsp70 promoter.

Mice were screened by Southern blot and/or PCR performed on tail DNA according to standard techniques. PCR was performed with the following primers: hGHL:GTGCAGTTCCTCAGGAGTGT; hGHR: CGAACTTGCTGTAGGTCTGC.

The amplification product was 171 bp long. Amplification conditions (35 cycles) were: 94°C for 20 sec, 58°C for 30 sec and 72°C for 20 sec. Heterozygous males and females were crossed and the homozygous progeny was identified by Southern blot, based on the intensity of the transgenic bands; their homozygosity was confirmed by checking the offspring when the homozygous male was mated to a non-transgenic partner. The mice used for the in vitro and in vivo experiments were always derived from a homozygous male bred with a non-transgenic CD-1 female.

Total RNA was extracted from different tissues (liver, spleen, lung, kidney, blood) of transgenic and control mice, according to standard techniques (Sambrook et al., "Molecular cloning: a laboratory manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Southern and Northern blot were performed according to standard techniques.

In order to evaluate the basal value of non-induced expression of the transgene, mice were analysed with Northern blot and with RT-PCR.

No expression was detected in lung, kidney, spleen, liver and peripheral blood lymphocytes of non-treated animals or of animals not-exposed to heat shock. The hGH

level in non-treated mice (control) was generally under the test detection limits, and when it was determined, it never exceeded 10 pg/ml.

EXAMPLE 2

In vivo heat shock treatment.

Eight transgenic mice obtained according to example 1 and four non-transgenic control mice were subjected to in vivo heat shock at 44°C for 30 min. Six additional unexposed transgenic mice were tested. Aliquots of blood were taken before and 1, 3, 5, 7, and 24 hours after the heat shock.

In transgenic mice (Fig. 2) a specific increase of plasma hGH was detected with a peak three hour after treatment.

These results suggest that the integrated transgene does not affect in vivo the normal responsiveness of hsp promoter.

EXAMPLE 3

a) Inducibility of the hsp70/hGH transgene expression in vivo by sodium arsenite and methylmercurium chloride.

Male transgenic mice obtained as described in example 1 were weighed, anesthetized with ether and injected intraperitoneally (i.p.) with $NaAsO_2$ dissolved in PBS, at a final dose of 2.5 or 5 mg/kg, or with 3.5 mg/kg CH_3HgCl dissolved in PBS. Control transgenic mice were injected with the same volume of PBS (about 200 µl/mouse).

Blood samples were recovered before injection and 1, 3, 5, 7 and 24 hours after treatment.

hGH plasma levels at different times and doses are shown in Fig. 3.

Both the tested doses of $NaAsO_2$ gave a clear and statistically significant response.

The response peaked after 3-5 hours and turned to the basal level 24 hours after injection.

CH_3HgCl gave hGH peaks after 5-7 hours and baseline hGH values 24 hours after injection.

b) Following the same procedure as described in a), hGH inducibility was evaluated in mice treated with rubidium chloride (18.5 mg/kg, c), copper sulfate (9 mg/kg, d) and cadmium chloride (4.7 mg/kg, e).

Results are reported in Fig. 3.

EXAMPLE 4

Inducibility of the hsp70/hGH transgene expression in vivo by repeated injections of toxic compounds.

Initially, 13 mice were treated as follows:

5 mice with As, 3 mice with Cd, 2 mice with Rb, 3 mice with Cu. After a period of 10 days to 2 months, the former three groups of mice were re-inoculated with As, the latter with Cu.

Blood samples were taken before and 3-5 hours after injection, i.e. at the times of highest response.

As shown in Fig. 4, after the first administration of the compound, the mice showed a response comparable to that observed in groups of mice treated as in example 3.

When retested after 10-60 days, a similar hGH increase was observed.

EXAMPLE 5

Embryonic fibroblast primary cultures-in vitro toxicity tests.

Homozygous transgenic mice obtained as described in

example 1 were crossed with CD-1 females. After 14 days, embryonic fibroblasts (EMFIS) were recovered from the fetuses according to the technique described by Robertson E.J., IRL Press, Oxford, 77-88, 1987.

Cells were cultured in DMEM supplemented with 10% FCS and antibiotics (pen/strep), in an incubator (CO_2:5%, 100% humidity). Culture medium was replaced every second day with pre-warmed (37°C) fresh culture medium. The cells were expanded for two passages and then frozen at -80°C. For each experiment, cells were thawed, plated in 10 cm Petri dishes, left to grow and then re-seeded on 12 well plates until confluence.

To evaluate the toxic effect of the compounds, cells were treated by substituting the culture medium with fresh pre-warmed serum-free medium containing the toxic compounds at the chosen final dilutions. Cells were exposed to the toxic compound for either 5 or 24 hours and then the medium was replaced with fresh control medium for an additional 24 hours. At the end of the treatment, culture media were collected and assayed for hGH secretion by enzyme immunoassay.

Each treatment was performed in triplicate and the hGH determination was repeated twice for each plate. The results are expressed as pg of hGH/10^6 cells. The sensitivity of this method was approximately 2-4 pg/ml.

As shown in the table, calcium and rubidium, known for their lack of toxicity at the tested concentrations, do not provoke hGH release in the medium.

On the contrary, a significant release is induced after 24 hours of chrome exposure, while copper gives a low response after 24 hours at the highest

concentrations. On the contrary, mercurium does not induce hGH release from fibroblasts at each tested concentration.

Finally, arsenic and cadmium, as expected, showed clearly toxic.

EXAMPLE 6

Primary hepatocytes cultures-in vitro toxicity tests.

Transgenic male mice 8 weeks old were anesthetized and their livers were perfused as described in Clerici et al., Mut. Res., 227:47-51, 1989, in order to collect hepatocytes. Hepatocytes were then seeded on 24 well plates ($2x10^5$ cells/well) and cultured in William's E medium supplemented with antibiotics (pen/strep) and 10% FCS for 2 hours in order to allow them to attach to the bottom of the Petri dishes. The supernatant was then removed and the adherent cells were treated with the compounds dissolved in the medium.

To evaluate the toxic effect of the compounds, cells were treated by substituting the culture medium with fresh pre-warmed serum-free medium containing the toxic compounds at the chosen final dilutions.

As shown in the table, calcium and rubidium do not induce hGH release by mature hepatocytes.

Chrome treatment induces a high response after 24 hours, while copper treatment causes release either after 5 or 24 hours at each concentration.

Mercurium induces a response at concentrations higher than $5x10^{-5}$ M, while arsenic and cadmium show extremely toxic.

EXAMPLE 7

In vitro toxicity tests on kidney, lung and bone-marrow primary cultures.

Kidney and lung cells were recovered as described by Campbell, J. A. et al. ("Sister cromatid exchange analysis of mice following in vitro exposure to vinyl carbonate", In vitro Cell. Dev. Biol. 22: 443:448, 1986).

Briefly, kidneys were removed from the same animals subjected to liver perfusion, washed 3 times in PBS additioned with antibiotics and minced in 0.5 mm pieces with a sterile scalpel. After 1 hour of incubation in trypsin/collagenase (100U/ml) solution, the suspension was centrifuged twice for 5 min. at 50xg, plated in 100 mm Falcon dishes and cultured in McCoy's medium with 20% FCS, 2mM Glutamine and Pen/strep.

In order to collect lung cells, after liver perfusion the chest cavity was opened after liver perfusion to access the lungs. The trachea was cut with a scalpel and a 22-gauge catheter was inserted into the trachea to perfuse the lungs with trypsin/collagenase solution for 5 min. in order to help the disaggregation of this tissue. The cells were then trypsinized, seeded in 24 wells and left to grow until confluence in McCoy's medium with 20% FCS, 2mM Glutamine and antibiotics.

In order to prepare bone marrow primary cultures, bone marrow cells were flushexd from the cavity of femurs and tibias with a syringe containing the culture medium. Cells were plated in 12 well plates with McCoy's medium with 20% FCS, 2mM Glutamine and antibiotics, and left to grow until the stromal cells reached confluence.

To evaluate the toxic effect of the compounds, the

same procedure was followed as in the above examples 5 and 6.

Results are reported in the Table.

Table

(A) Determination of hGH (pg/10^6 cells) release and primary transgenic cultures viability after 5-hour treatment

Compounds	Primary lines	hGH release 10^{-5}M	$5x10^{-5}$M	10^{-4}M	$5x10^{-4}$M	Viabilty 10^{-5}M	$5x10^{-5}$M	10^{-4}M	$5x10^{-4}$M
$CaCl_2$	hepatocytes	nd	nd	nd	nd	+	+	+	+
RbCl		nd	nd	nd	nd	+	+	+	+
$CrCl_3$		/	nd	nd	nd	/	+	+	+
$CuSO_4$		/	nd	80	66	/	+	+	+
K_2Cr2O_7		nd	65	94	65	+/-	+/-	-	-
CH_3HgCl		nd	nd	nd	/	+/-	+/-	-	/
$CdCl_2$		309	452	57	14	+/-	+/-	-	-
$NaAsO_2$		100	224	nd	/	+	+/-	-	/
$CaCl_2$	Embryonic	/	nd	nd	nd	/	+	+	+
RbCl	fibroblast	/	nd	nd	nd	/	+	+	+
$CrCl_3$		/	nd	nd	nd	/	+	+	+
$CuSO_4$		/	nd	6	12	/	+	+	+
K_2Cr2O_7		9	nd	nd	nd	+/-	+/-	-	-
CH_3HgCl		/	nd	nd	nd	/	+/-	-	/
$CdCl_2$		250	85	45	nd	+/-	+/-	-	-
$NaAsO_2$		nd	113	19	nd	+	+/-	+/-	/

continues

$CaCl_2$	Kidney cells	/	nd	nd	nd	/	+	+	+
RbCl		/	57	nd	nd	/	+	+	+
$CrCl_3$		/	nd	15	nd	/	+	+	+/-
$CuSO_4$		/	nd	nd	nd	/	+	+/-	+/-
$K_2Cr_2O_7$		nd	nd	nd	/	+/-	+/-	+/-	/
CH_3HgCl		10	nd	nd	/	+/-	+/-	+/-	/
$CdCl_2$		nd	nd	nd	/	+/-	-	-	/
$NaAsO_2$		22	17	28	/	+	+/-	-	/

$CaCl_2$	Lungs cells	/	nd	127	202	/	+	+	+
RbCl		/	28	191	71	/	+	+	+
$CrCl_3$		/	92	122	166	/	+	+	+
$CuSO_4$		/	nd	nd	184	/	+/-	+/-	+/-
$K_2Cr_2O_7$		nd	nd	nd	/	+/-	-	-	/
CH_3HgCl		27	nd	nd	/	-	-	-	/
$CdCl_2$		nd	31	11	/	+/-	-	-	/
$NaAsO_2$		nd	37	249	/	+/-	+/-	-	/

hGH levels in untreated cells medium (controls) were not measurable after 5-24-hour incubation.

nd = undetectable; / = not determined; + = with 100% viability; with 30-70% viability;

- = 100% dead

(B) Determination of hGH (pg/10^6 cells) release and primary transgenic coltures after 24-hour treatment.

Compounds	Primary lines	hGH release 10^{-5}M	hGH release $5x10^{-5}$M	hGH release 10^{-4}M	hGH release $5x10^{-4}$M	Vitality 10^{-5}M	Vitality $5x10^{-5}$M	Vitality 10^{-4}M	Vitality $5x10^{-4}$M
$CaCl_2$	hepatocytes	nd	nd	nd	nd	+	+	+	+
RbCl		nd	nd	nd	nd	+	+	+	+
$CrCl_3$		/	36	20	nd	/	+	+	-
$CuSO_4$		/	12	61	100	/	+	+	+/-
$K_2Cr_2O_7$		nd	nd	nd	nd	-	-	-	-
CH_3HgCl		nd	63	103	/	+/-	-	-	/
$CdCl_2$		nd	nd	17	21	+/-	+/-	-	-
$NaAsO_2$		270	19	5	/	+	+/-	-	/
$CaCl_2$	Embryonic fibroblast	/	nd	nd	nd	/	+	+	+
RbCl		/	nd	nd	nd	/	+	+	+
$CrCl_3$		/	8	10	6	/	+	+	+
$CuSO_4$		/	nd	10	47	/	+	+	+
$K_2Cr_2O_7$		nd	nd	nd	nd	+/-	-	-	-
CH_3HgCl		/	nd	nd	nd	/	-	-	-
$CdCl_2$		181	108	41	nd	-	-	-	-
$NaAsO_2$		19	380	37	4	+/-	+/-	-	-

continues

$CaCl_2$	Kidney	/	nd	nd	nd	/	+	+	+
RbCl	cells	/	nd	nd	nd	/	+	+	+
$CrCl_3$		/	nd	nd	nd	/	+	+	+/-
$CuSO_4$		/	nd	nd	450	/	+/-	+/-	-
$K_2Cr_2O_7$		nd	nd	nd	/	-	-	-	/
CH_3HgCl		nd	nd	nd	/	-	-	-	/
$CdCl_2$		nd	81	110	/	+/-	-	-	/
$NaAsO_2$		300	nd	40	/	+	+/-	-	/
$CaCl_2$	Lungs	/	nd	nd	nd	/	+	+	+/-
RbCl	cells	/	20	110	114	/	+	+	+/-
$CrCl_3$		/	200	199	35	/	+/-	+/-	+/-
$CuSO_4$		/	81	132	901	/	+/-	+/-	-
$K_2Cr_2O_7$		13	92	nd	/	-	-	-	/
CH_3HgCl		nd	164	nd	/	-	-	-	/
$CdCl_2$		64	196	415	/	+/-	-	-	/
$NaAsO_2$		20	55	nd	/	+/-	-	-	/
$CaCl_2$	Bone marrow	/	nd	51	nd	/	+	+	+/-
RbCl	cells	/	nd	20	128	/	+	+	+/-
$CrCl_3$		/	nd	21	21	/	+	+	+/-
$CuSO_4$		/	nd	127	145	/	+	+	-
$K_2Cr_2O_7$		nd	38	127	/	+/-	-	-	/
CH_3HgCl		nd	42	165	/	+/-	-	-	/
$CdCl_2$		nd	nd	nd	/	+/-	-	-	/
$NaAsO_2$		nd	nd	nd	/	+	+/-	+/-	/

hGH levels in untreated cells medium (controls) were not measurable after 24-hour incubation.
nd = undetectable; / = not determined; + = with 100% viability; with 30-70% viability;
- = 100% dead

CLAIMS

1. A non-human transgenic mammal which comprises cells containing a construct of a heat shock protein (hsp) promoter linked to the growth hormone (GH) gene sequence.

2. A non-human transgenic mammal according to claim 1, wherein the heat shock protein promoter is hsp70 gene promoter.

3. A non-human transgenic mammal according to claim 1, which is a rodent.

4. A non-human transgenic mammal according to claim 3, which is a mouse.

5. A method for the study of chemical, physical and biological toxic agents which comprises:

a) exposing the transgenic mammal of claims 1-4 to the toxic agent;

b) determining the effect through measurement of the hematic concentration of the reporter-gene.

6. A method according to claim 5, wherein the same animal is used for repeated tests with the same or different toxic agent.

7. A method according to claims 5-6, for the study of toxicity kinetics of one or more toxic agents.

8. A method according to claims 5-6, for the study of heat stress.

9. A method according to claims 5-6, for the study of metal toxicity.

10. A method according to claim 9 for the study of toxicity of metals selected from the group consisting of Rb, Cu, Hg, As and Cd.

11. The use of the transgenic mammal of claim 1 for in vivo toxicity studies.

12. The use of a transgenic animal according to claim 11, wherein said animal is a mouse.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A1

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(30) **Priority Data:**
MI97A001972 28 August 1997 (28.08.97) IT

(71) **Applicant** *(for all designated States except US)*: CONSIGLIO NAZIONALE DELLE RICERCHE [IT/IT]; Piazzale Aldo Moro, 7, I–00185 Roma (IT).

(72) **Inventor:** RONCUCCI, Romeo (deceased).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: SACCO, Maria, Grazia [IT/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT). ZECCA, Luigi [IT/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT). BROMLEY, Peter [CH/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT). CLERICI, Libero, A. [IT/IT]; Joint Research Center, Environment Institute, I–21027 Ispra (IT). VEZZONI, Paolo [IT/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT).

(74) **Agent:** MINOJA, Fabrizio; Bianchetti Bracco Minoja S.r.l., Via Rossini, 8, I–20122 Milano (IT).

(81) **Designated States:** AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) **Title:** TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS

(57) **Abstract**

The invention provides non–human transgenic animals bearing regulatory DNA sequences in some or all their cells, which are sensitive to biological, physical and chemical toxic agents. Such sequences are linked to sequences of reporter genes useful for toxicological studies.

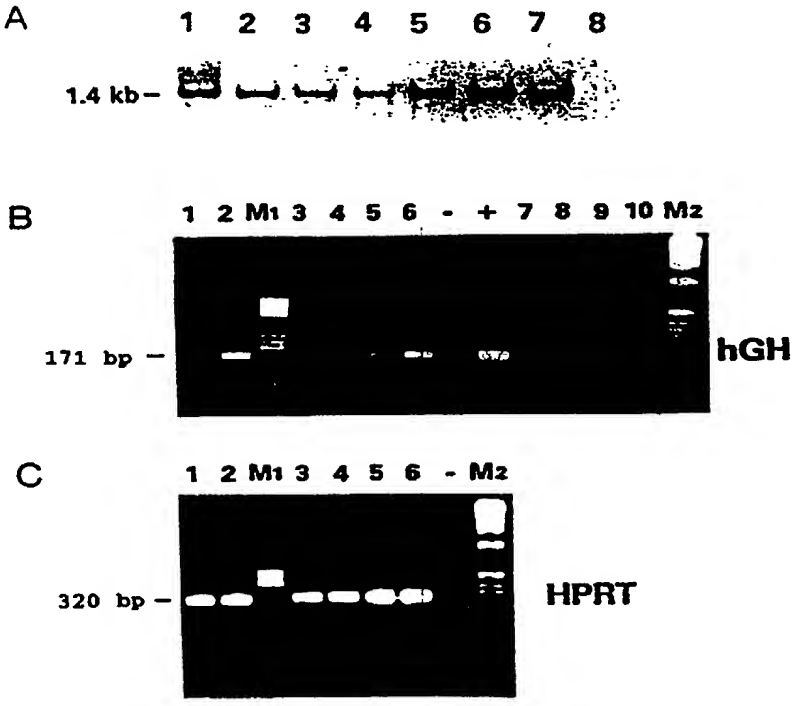
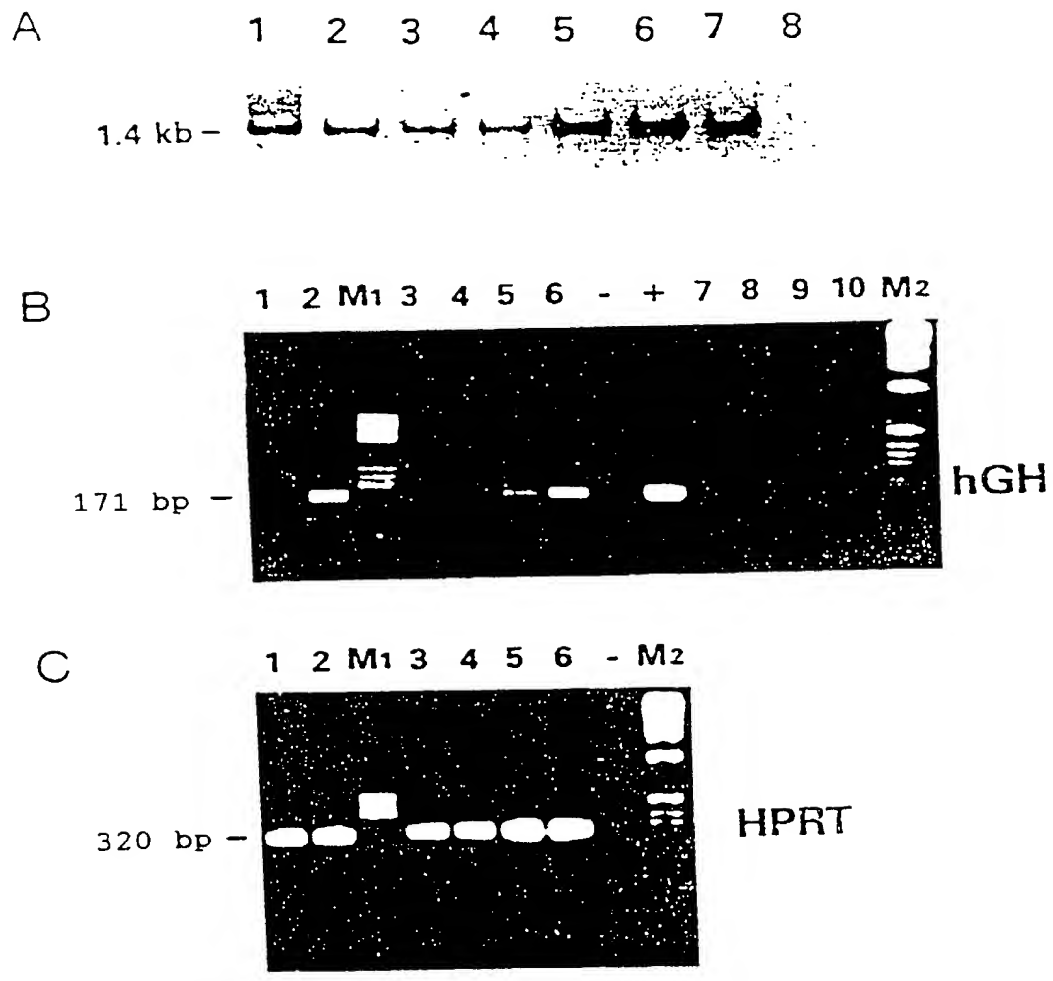


FIGURE 1


A
1 2 3 4 5 6 7 8
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B
1 2 M1 3 4 5 6 - + 7 8 9 10 M2
171 bp –
hGH
C
1 2 M1 3 4 5 6 - M2
320 bp –
HPRT

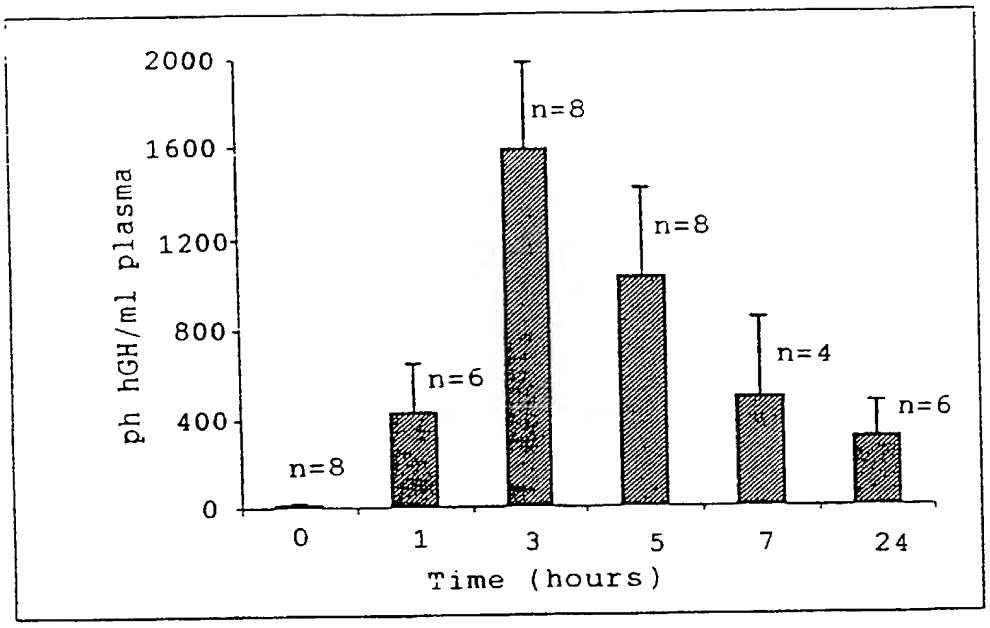

2000
1600
1200
800
400
0
ph hGH/ml plasma
n=8
n=6
n=8
n=8
n=4
n=6
0
1
3
5
7
24
Time (hours)

FIGURE 2

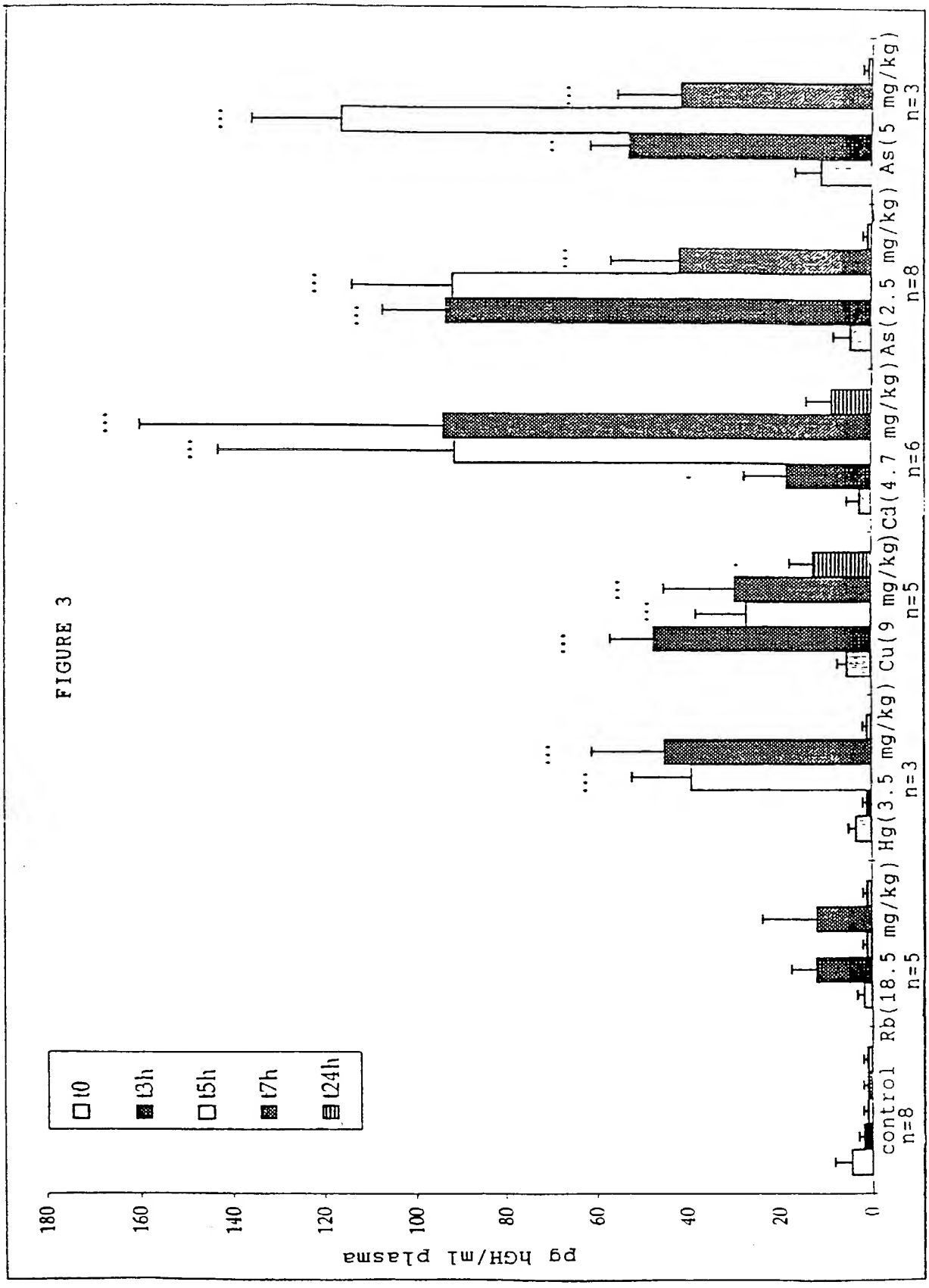
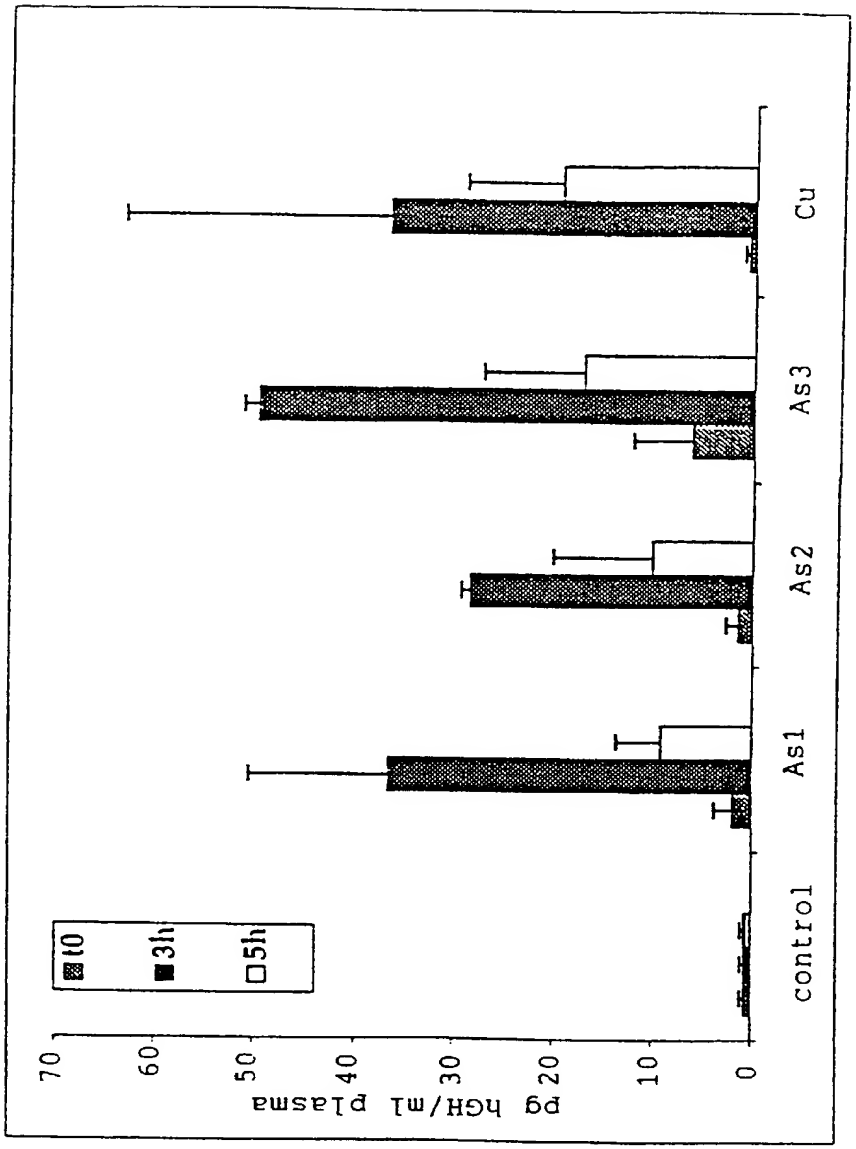

pg hGH/ml plasma
0
20
40
60
80
100
120
140
160
180
t0
t3h
t5h
t7h
t24h
control n=8
Rb(18.5 mg/kg) n=5
Hg(3.5 mg/kg) n=3
Cu(9 mg/kg) n=5
Cd(4.7 mg/kg) n=6
As(2.5 mg/kg) n=8
As(5 mg/kg) n=3

FIGURE 3

FIGURE 4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As1
As2
As3
Cu
t0
3h
5h
pg hGH/ml plasma
0
10
20
30
40
50
60
70

DECLARATION FOR UNITED STATES PATENT APPLICATION
(For Use With Both PCT and Non-PCT Applications) (Atty. Docket: SCBREV-223)

As a below named inventor, I declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled, (1) TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS, the specification of which is attached hereto unless the following box is checked: (2) [xx] was filed on (3) 28 February 2000 (4) as U. S. Appl. SN or PCT International Appl. No. _____ and was amended on (5) 28 February 2000 (if applicable).

I state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

	NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
(6)	MI97A 001972	ITALY	28 August 1997	[X] Yes [ ] No
	PCT/IT98/00231	PCT	11 August 1998	[X] Yes [ ] No
				[ ] Yes [ ] No
				[ ] Yes [ ] No

I claim the benefit under 35 USC 120 of the United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

	Appln. Serial No.	Filing Date	
(7)			(Status: [ ] Patented [ ] Pending [ ] Abandoned)
			(Status: [ ] Patented [ ] Pending [ ] Abandoned)

I appoint B. Franklin Griffin, Jr., Reg. No. 19,334; F. Prince Butler, Reg. No. 25,666; Fred S. Whisenhunt, Reg. No. 24,378; Joerg-Uwe Szipl, Reg. No. 31,799; and Richard J. Gallagher, Reg. No. 28,781, individually and jointly my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and with the resulting patent, whose address is Griffin, Butler, Whisenhunt & Szipl, LLP, Suite PH-1, 2300 9th Street, South, Arlington, Virginia 22204-2320, Telephone No. (703) 979-5700, Customer No. 113.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(8) Full name of sole or first inventor SACCO, Maria Grazia
Inventor's signature _____ Date 21 March 2000
Residence Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, 20131 Milano, Italy
Citizenship Italian
Post Office Address Same as Above

(8) Full name of joint inventor ZECCA, Luigi
Inventor's signature _____ Date 23 March 2000
Residence Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, 20131 Milano, Italy
Citizenship Italian
Post Office Address Same as Above

(8) Full name of joint inventor BROMLEY, Peter
Inventor's signature _____ Date _____
Residence Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, 20131 Milano, Italy
Citizenship Switzerland
Post Office Address Same as Above

(1) Insert title of invention.
(2) Check block for PCT application or U. S. application already on file, and complete items (3), (4) and (5). If PCT national phase entry application, insert international PCT application filing date, Serial No., and date of any Article 19 amendments.
(6) Complete for foreign priority documents; add additional page if needed.
(7) Complete for earlier US parent applications; add additional page if needed.
(8) Complete ALL blanks. Attach second page for further joint inventors.

CAUTION: THIS FORM MAY BE USED ONLY IF ALL INVENTORS READ AND UNDERSTAND ENGLISH.

corrected 31/10/2000

300 ✓

(8) Full name of joint inventor CLERICI, Libero
Inventor's signature [signature] Date 23 March 2000 ✓
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Post Office Address Same as Above

400 ✓

(8) Full name of joint inventor VEZZONI, Paolo
Inventor's signature [signature] Date 17 March, 2000 ✓
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Post Office Address Same as Above

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Inventor's signature Date
Residence Citizenship
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500

(8) Full name of joint inventor BROMLEY PETER
Inventor's signature Peter Bromley Date 31, March, 00
Residence GENE CONTROL S.A. 9, RUE BOISSONNAS, P.O. BOX 227 CH-1211, GENEVA 24, SWITZERLAND Citizenship BRITISH
Post Office Address CITY

(8) Full name of joint inventor
Inventor's signature Date
Residence Citizenship
Post Office Address

(8) Full name of joint inventor
Inventor's signature Date
Residence Citizenship
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(8) Full name of joint inventor
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Post Office Address

(8) Full name of joint inventor
Inventor's signature Date
Residence Citizenship
Post Office Address

8386

DECLARATION FOR UNITED STATES PATENT APPLICATION
(For Use With Both PCT and Non-PCT Applications) (Atty. Docket: SCSREV-223)

As the below named legal guardian of Rachele RONCUCCI and Régine RONCUCCI, I declare that Rachele RONCUCCI and Régine RONCUCCI are minor heirs of Romeo RONCUCCI, deceased, who is an inventor/applicant of the subject matter which is claimed and for which a patent is sought on the invention entitled, (1) TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS, the specification of which is attached hereto unless the following box is checked: (2) [XX] was filed on (3) 28 February 2000 (4) as U. S. Appl. SN or PCT International Appl. No. _____ and was amended on (5) 28 February 2000 (if applicable).

I state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. The duty to disclose information which is material to patentability as defined in 37 CFR 1.56 is hereby acknowledged. Foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below are hereby claimed and identified below are any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

	NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
(6)	MI97A 001972	ITALY	28 August 1997	[X] Yes [ ] No
	PCT/IT98/00231	PCT	11 August 1998	[X] Yes [ ] No
				[ ] Yes [ ] No
				[ ] Yes [ ] No

I claim the benefit under 35 USC 120 of the United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

	Appln. Serial No.	Filing Date	
(7)			(Status: [ ] Patented [ ] Pending [ ] Abandoned)
			(Status: [ ] Patented [ ] Pending [ ] Abandoned)

I appoint B. Franklin Griffin, Jr., Reg. No. 19,334; F. Prince Butler, Reg. No. 25,666; Fred S. Whisenhunt, Reg. No. 24,378; Joerg-Uwe Szipl, Reg. No. 31,799; and Richard J. Gallagher, Reg. No. 28,781, individually and jointly my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and with the resulting patent, whose address is Griffin, Butler, Whisenhunt & Szipl, LLP, Suite PH-1, 2300 9th Street, South, Arlington, Virginia 22204-2320, Telephone No. (703) 979-5700, Customer No. 113.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

601 Romeo RONCUCCI, Deceased

BY: Maria Novella Castagnoli
Maria Novella CASTAGNOLI, Guardian of Rachele RONCUCCI and Régine RONCUCCI, who are minor heirs of Romeo RONCUCCI, Deceased, and who reside at via Ungaretti 17, 20028 San Vittore Olona, Milano, Italy

701

IT X

Dated: 23.05, 2000

(1) Insert title of invention.
(2) Check block for PCT application or U. S. application already on file, and complete items (3), (4) and (5). If PCT national phase entry application, insert international PCT application filing date, Serial No., and date of any Article 19 amendments.
(6) Complete for foreign priority documents; add additional page if needed.
(7) Complete for earlier US parent applications; add additional page if needed.

CAUTION: THIS FORM MAY BE USED ONLY IF ALL INVENTORS READ AND UNDERSTAND ENGLISH.

09486660 .110299

8386

DECLARATION FOR UNITED STATES PATENT APPLICATION
(For Use With Both PCT and Non-PCT Applications) (Atty. Docket: SCHREV-323)

I declare that I am the below named heir of Romeo RONCUCCI, deceased, who is an inventor/applicant of the subject matter which is claimed and for which a patent is sought on the invention entitled, (1) TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS the specification of which is attached hereto unless the following box is checked: (2) [XX] was filed on (3) 28 February 2000 (4) as U. S. Appl. SN or PCT International Appl. No. _____ and was amended on (5) 28 February 2000 (if applicable).

I state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. The duty to disclose information which is material to patentability as defined in 37 CFR 1.56 is hereby acknowledged. Foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below are hereby claimed and identified below are any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

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	PCT/IT98/00231	PCT	11 August 1998	[X] Yes [ ] No
				[ ] Yes [ ] No
				[ ] Yes [ ] No

I claim the benefit under 35 USC 120 of the United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

	Appln. Serial No.	Filing Date	
(7)			(Status: [ ] Patented [ ] Pending [ ] Abandoned)
			(Status: [ ] Patented [ ] Pending [ ] Abandoned)

I appoint B. Franklin Griffin, Jr., Reg. No. 19,334; F. Prince Butler, Reg. No. 25,666; Fred S. Whisenhunt, Reg. No. 24,378; Joerg-Uwe Szipl, Reg. No. 31,799; and Richard J. Gallagher, Reg. No. 28,781, individually and jointly my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and with the resulting patent, whose address is Griffin, Butler, Whisenhunt & Szipl, LLP, Suite PH-1, 2300 9th Street, South, Arlington, Virginia 22204-2320, Telephone No. (703) 979-5700, Customer No. 113.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

807 Romeo RONCUCCI, Deceased

BY: _____
Sylvia RONCUCCI, an heir of Romeo RONCUCCI, Deceased, residing at Via Thaon di Revel, 12, 20159 Milano, Italy

IX

Dated: 23.05, 2000

(1) Insert title of invention.
(2) Check block for PCT application or U. S. application already on file, and complete items (3), (4) and (5). If PCT national phase entry application, insert international PCT application filing date, Serial No., and date of any Article 19 amendments.
(6) Complete for foreign priority documents; add additional page if needed.
(7) Complete for earlier US parent applications; add additional page if needed.

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